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Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins

(glycated protein/endothelium/receptor/diabetes mellitus)

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ABSTRACT **The extended interaction of aldoses with proteins or lipids results in nonenzymatic glycation and oxidation, ultimately forming AGEs, the presence of which in the plasma and vessel wall is associated with diabetic vascular complications. We show here that AGE albumin in the intravascular space interacts with the vessel wall via binding to an integral membrane protein, receptor for AGE (RAGE), a member of the immunoglobulin superfamily, resulting in clearance from the plasma and induction of interleukin 6 mRNA. Intravenously infused ^{125}I-AGE albumin showed a rapid phase of plasma clearance with deposition in several organs. Rapid removal of ^{125}I-AGE albumin from the plasma was prevented by administration of a soluble, truncated form of RAGE, which blocked binding of ^{125}I-labeled AGE albumin to cultured endothelial cells and mononuclear phagocytes, as well as by pretreatment with anti-RAGE IgG. Ultrastructural studies with AGE albumin–colloidal gold conjugates perfused *in situ* showed that in murine coronary vasculature this probe was taken up by endothelial plasmalemmal vesicles followed by transport either to the abluminal surface or by accumulation in intracellular vesicular structures reminiscent of endosomes and lysosomes. Consequences of AGE–RAGE interaction included induction of interleukin 6 mRNA expression in mice. These data indicate that RAGE mediates the interaction of AGEs with the vessel wall, both for removal of these glycated proteins from the plasma and for changes in gene expression.**

When proteins or lipids are exposed to aldoses, they undergo nonenzymatic glycation and oxidation (1–8), ultimately forming AGEs, whose formation occurs during normal aging and is accelerated in diabetics (1–7). The presence of AGEs in the plasma and vessel wall has been linked to the pathogenesis of diabetic complications, stimulating investigations to determine mechanisms through which AGEs exert their pathologic effects.

An important mechanism through which AGEs interact with cells is through specific receptors (9–13). We thus evaluated the role of the receptor for AGE (RAGE), which specifically binds AGEs (10–12), in mediating the interactions of these glycated molecules with target cells such as endothelial cells (ECs) and mononuclear phagocytes (MPs) (10, 11, 13). Previous studies have identified the presence of RAGE in bovine cardiac vasculature (13). We have now identified RAGE in murine coronary vasculature both *in vivo* and *in vitro* and employed this model to demonstrate that RAGE has a central role in uptake by the endothelium and in gene expression following AGE infusion.

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MATERIALS AND METHODS

Preparation of AGE Albumin, RAGE, and Anti-RAGE Antibody. Mouse and bovine AGE albumin were prepared and characterized as described (2, 10, 11). Radiolabeling of AGE and native albumin was performed by the lactoperoxidase method (14); the tracers had specific radioactivities of $\approx 1.5 \times 10^4$ cpm/ng (10, 11). For AGE albumin–gold conjugates, colloidal gold particles (5 nm in diameter) were prepared as described (15, 16). Bovine RAGE and monospecific rabbit anti-RAGE IgG were prepared and characterized as described (11). The ≈35-kDa form of bovine RAGE was termed soluble RAGE (sRAGE; refs. 10 and 12).

Cell Binding Assays. Binding of ^{125}I-AGE albumin to cultured bovine adrenal capillary ECs or human MPs was studied as described (10, 11, 17).

Infusion/Uptake Studies. ^{125}I-labeled AGE albumin or ^{125}I-labeled native albumin (≈3 μg) was infused via the tail vein of CD_1 mice with or without preincubation with a 50-fold molar excess of sRAGE or preinfusion of the animals with either anti-RAGE IgG or nonimmune IgG. To assess tissue deposition, organs were removed and the weight and radioactivity (cpm) were measured (18). The method of Spady *et al.* (19) was used to calculate the tissue spaces. To correct for nonspecific tissue trapping of tracer, a tissue space for ^{125}I-albumin was calculated (18). All preparations of proteins utilized in infusion studies were tested in the *Limulus* amebocyte assay (Sigma) for lipopolysaccharide content and, where indicated, were chromatographed on Detoxi-Gel columns (Pierce). Inactive heat-treated AGE albumin (boiled for 15 min) was also employed.

Effect of AGE Albumin Infusion on Levels of Interleukin 6 (IL-6) Transcripts. Three hours after AGE albumin infusion, mice were sacrificed, livers were excised, total RNA was extracted, and poly(A)$^+$ mRNA was prepared (20, 21). Random hexanucleotide-primed first-strand cDNA served as the template for polymerase chain reaction (PCR) analysis. Murine IL-6 primers and actin primers were obtained from Clontech. cDNA for murine IL-6 was amplified by PCR for 35 cycles, each consisting of incubations at 94°C for 2 min (first cycle) or 45 sec (remaining 34 cycles), 60°C for 45 sec, and 72°C for 2 min followed by 7 min at 72°C linked to 4°C. A similar protocol was utilized for the β-actin primers except that 25 cycles were employed. Products were separated by 2% agarose gel electrophoresis and transferred to nylon membrane for Southern hybridization with a ^{32}P-labeled oligonucleotide probe for murine IL-6 (Clontech).

Abbreviations: AGE, advanced glycation end product; RAGE, receptor for AGE; sRAGE, soluble RAGE; EC, endothelial cell; MP, mononuclear phagocyte; IL, interleukin.

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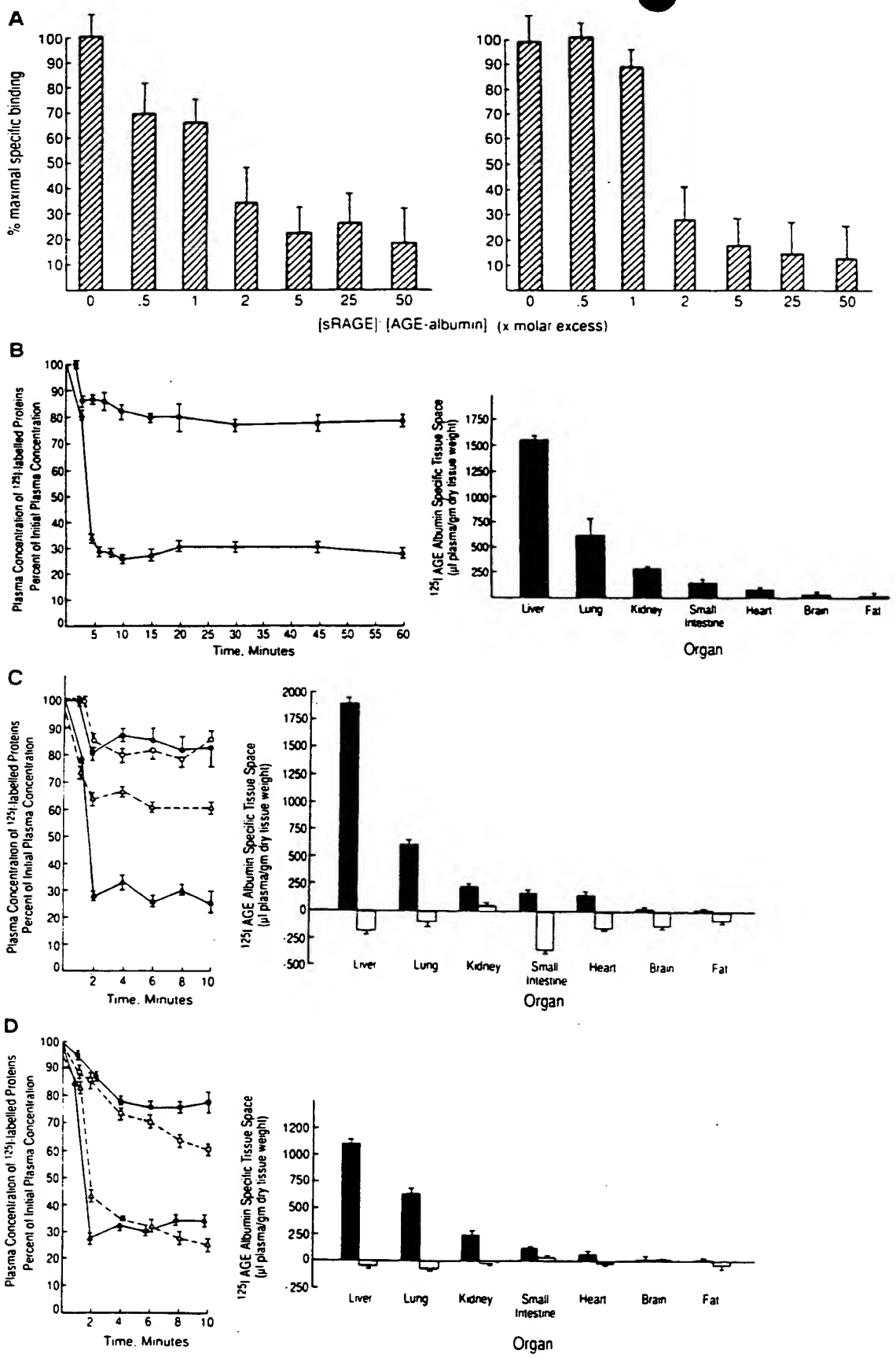

FIG. 1. *(Legend appears at the bottom of the opposite page.)*

Ultrastructural Studies of Vessel Uptake of AGE Albumin. Via laparotomy, a rtae were catheterized, and the vasculature was washed free of blood with phosphate-buffered saline (PBS) containing albumin (3.5 mg/ml) by using the vena cava as the outflow track. AGE albumin conjugated to colloidal gold was introduced at a flow rate of 3 ml/min at 37°C for 4 r 15 min. Unbound ligand was removed by perfusing PBS (3 min; 3 ml/min) and then 2.5% formaldehyde/1.5% glutaraldehyde/2.5 mM $CaCl_2$/0.1 M sodium cacodylate-HCl buffer (pH 7.2). Thin sections of myocardium were cut on an OmU Reichert Ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Philips 400 HM electron microscope.

Identification of Murine RAGE Antigen and mRNA in Cardiac Vasculature and Cultured Murine Coronary ECs. RAGE was detected immunohistochemically on freshly harvested mouse cardiac tissue fixed overnight with 3.5% formalin in PBS and on confluent cultured murine coronary ECs (generously provided by R. Auerbach, University of Wisconsin, Madison) fixed in buffered 3.5% paraformaldehyde. After preparation, immunostaining with anti-RAGE IgG was performed as described (13). *In situ* hybridization was performed with digoxigenin-labeled RNA probes (13). Immunoblotting was performed with 10^7 cultured murine coronary ECs (10, 11, 13, 22).

RESULTS

RAGE Expression in Murine Cardiac Vasculature. Immunohistochemistry demonstrated the presence of RAGE in murine coronary vessels, compared with absence of staining with nonimmune IgG, and *in situ* hybridization confirmed the presence of the mRNA with antisense probe, whereas sense controls were negative (data not shown). Experiments were also performed with cultured murine coronary ECs: RAGE was evident in nonpermeabilized samples, and *in situ* hybridization demonstrated RAGE mRNA (data not shown). Consistent with these results, Western blotting of detergent extracts of cultured murine coronary ECs demonstrated the presence of a single band at ≈35 kDa which was specifically immunoreactive with anti-RAGE IgG.

Effect of sRAGE and Anti-RAGE IgG on the Binding of AGE Albumin to Cultured ECs and MPs and on the Removal of Infused AGE Albumin from the Blood. To analyze the contribution of RAGE in the interaction of AGEs with cellular elements, we employed monospecific polyclonal anti-RAGE IgG which blocks the interaction of AGEs with cultured ECs and MPs (10, 11), and sRAGE. Addition of sRAGE to incubation mixtures of ^{125}I-labeled AGE albumin with either ECs or MPs resulted in dose-dependent inhibition of binding (Fig. 1*A*). sRAGE did not affect binding of ^{125}I-labeled factor IX to cultured ECs (data not shown).

To determine t of RAGE in the handling of circulating AGEs, infusi n studies were performed with ^{125}I-labeled AGE or native albumin in mice (Fig. 1*B*) ^{125}I-labeled AGE albumin showed an initial rapid phase of removal from the blood, with ≈70% of the material gone by 5 min (Fig. 1*B Left*). The deposition of ^{125}I-AGE albumin in the tissues, studied just after the rapid phase of AGE clearance from the blood, was enhanced relative to that of ^{125}I-albumin, especially in the liver, lung, and kidney (Fig. 1*B Right*). When ^{125}I-AGE albumin was preincubated with sRAGE and infused into mice (Fig. 1*C*), the rapid phase of tracer clearance was largely blocked; sRAGE had no effect on ^{125}I-albumin plasma levels (Fig. 1*C Left*). Animals pretreated with anti-RAGE IgG showed an even more complete blockade of the early clearance phase of infused ^{125}I-AGE albumin. The clearance of ^{125}I-albumin was again unaffected (data not shown). Nonimmune IgG had no effect (Fig. 1*D Left*). Administration of ^{125}I-AGE albumin with sRAGE or pretreatment with anti-RAGE IgG prior to ^{125}I-AGE albumin infusion strikingly decreased its deposition in the organs (Figs. 1 *C Right* and *D Right*, respectively), suggesting that AGE albumin is initially cleared from the circulation and deposited in the organs/vasculature via a process which involves RAGE.

Ultrastructural Studies of AGE Albumin with the Vessel Wall. To assess vessel wall processing of infused AGEs, morphologic studies were performed with AGE albumin conjugated to colloidal gold particles, employing murine cardiac vasculature as a model system (23, 24). Four minutes after *in situ* perfusion, AGE albumin–gold conjugates decorated numerous plasmalemmal vesicles opened to the luminal front (Fig. 2*A*), while coated pits and coated vesicles were unlabeled (Fig. 2*B*). However, multivesicular bodies (Fig. 2*B*) and structures resembling endosomes appeared significantly decorated by the tracer (Fig. 2 *C* and *D*). Gold particles were also seen in the proximity of the abluminal endothelial cell surface (Fig. 2*D*). Sparse gold particles were occasionally observed in the subendothelial space (Fig. 2 *A* and *D*), suggesting that transcytosis had occurred. At 15 min, the presence of AGE albumin–gold particles in the subendothelial space was even more apparent. These experiments were performed in the presence of a large excess of native albumin (3.5 mg/ml) to block the interaction of determinants on albumin with vessel wall albumin-binding proteins (24–27). In addition, the presence of excess free AGE albumin blocked association of AGE albumin–gold particles with the heart tissue by >50%, while anti-RAGE IgG prevented such association by 50–70%. Similar results were observed in studies using ^{125}I-AGE albumin (data not shown).

Infusion of AGE Albumin Leads to Induction of IL-6 mRNA: Effect of Anti-RAGE IgG. AGEs have been reported t modulate cell properties *in vitro*, including the induction of genes for cytokines and growth factors (28, 29). In view of the

FIG. 1. Intravascular perfusion of ^{125}I-AGE albumin and ^{125}I-albumin into mice: Effect of sRAGE and anti-RAGE IgG. (*A*) Effect of sRAGE on the binding of ^{125}I-AGE albumin to cultured ECs (*Left*) and MPs (*Right*). (*Left*) Confluent bovine adrenal capillary ECs were incubated with ^{125}I- E albumin (100 nM) either alone (total binding), in the presence of a 30-fold excess of unlabeled AGE albumin (nonspecific binding), or w the indicated molar excess of sRAGE for 3 hr at 4°C. Percent maximal specific binding (total minus nonspecific binding), mean ± SEM of triplicate determinations, is shown. Maximal specific binding was ≈10 fmol per well. (*Right*) Cultured human MPs (5×10^4 per well) were tested for their capacity to bind ^{125}I-AGE albumin (100 nM) as in *A Left*. Maximal binding with MPs was ≈6 fmol per well. (*B Left*) Removal of ^{125}I-AGE albumin and ^{125}I-native albumin from the plasma. Mice were infused with tracer (3 μg per animal), and blood was withdrawn for determination of radioactivity. The mean ± SEM is shown, and experiments were repeated at least three times. (*B Right*) Deposition of infused ^{125}I-labeled AGE albumin (▲) or ^{125}I-labeled native albumin (●). Mice were infused as in *B Left* and after 10 min radioactivity in the tissues was determined. Results in mice treated with ^{125}I-AGE albumin were compared with those in animals exposed to ^{125}I-native albumin, and the mean ± SEM is shown. (*C*) Effect of sRAGE on ^{125}I-AGE albumin clearance from the plasma (*Left*) and deposition in the tissues (*Right*). The same experiment as in *B* was performed, but both ^{125}I-AGE albumin (▲, △) and ^{125}I-albumin (●, ○) were preincubated for 60 min at 37°C with (△, ○, open bars) or without (▲, ●, filled bars) a 50-fold molar excess of sRAGE. (*D*) Effect of anti (α)-RAGE IgG on ^{125}I-AGE albumin clearance from the plasma (*Left*) and deposition in the tissues (*Right*). The same experiment as in *B* was performed, but animals were pretreated for 30 min with either anti-RAGE IgG or nonimmune (NI) IgG (40 μg per animal). ▲ and filled bars, ^{125}I-AGE albumin; □ and open bars, ^{125}I-AGE plus anti-RAGE IgG; △, ^{125}I-AGE albumin plus nonimmune IgG; ●, ^{125}I-albumin. In all cases representative experiments are shown. Approximately 50 mice were employed for each experimental condition.

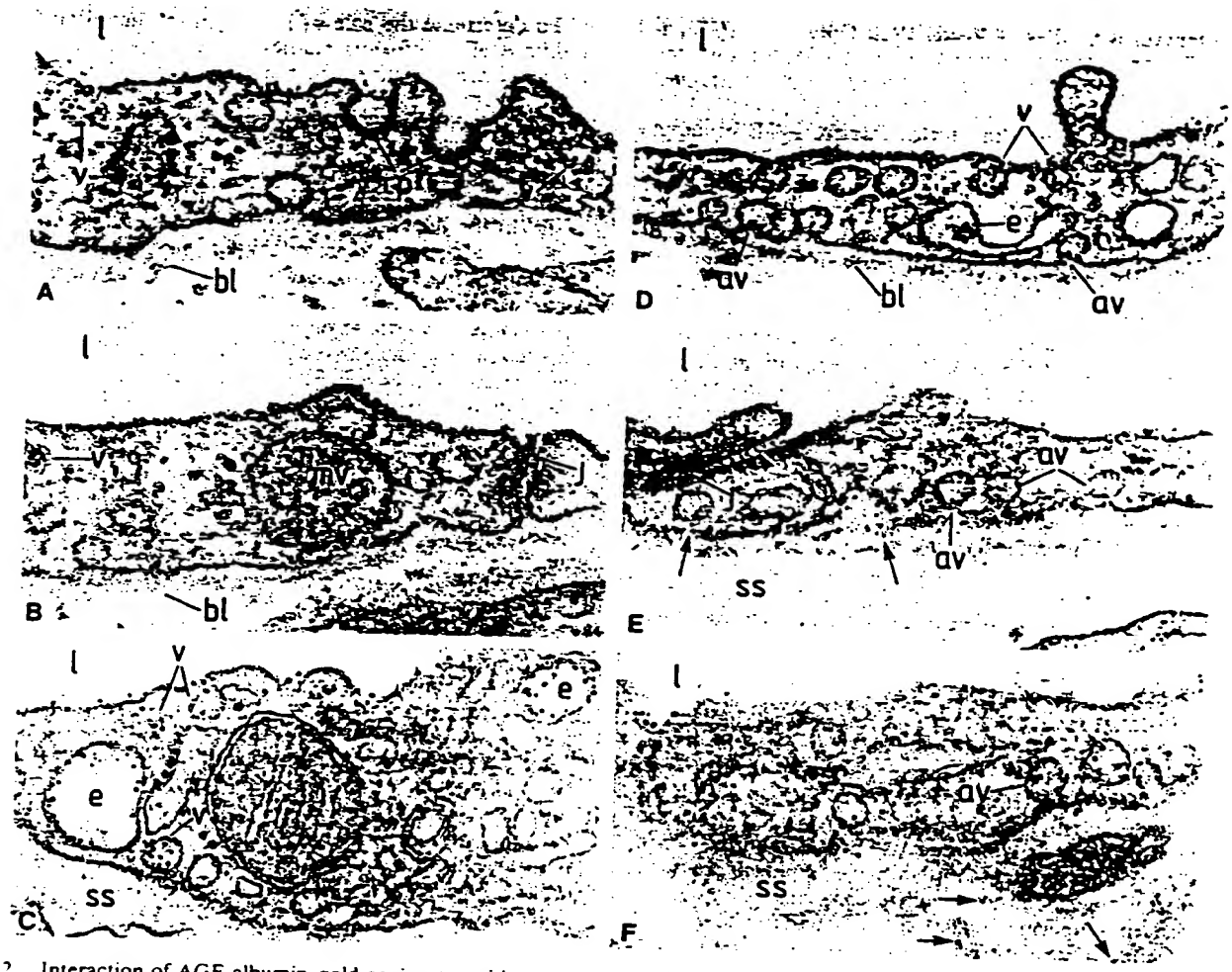

FIG. 2. Interaction of AGE albumin–gold conjugates with myocardial capillary endothelium at 4 min (*A–C*) and 15 min (*D–F*) after tracer perfusion *in situ*. (*A*) The particles are preferentially taken up by plasmalemmal vesicles (v), open to the luminal front, whereas coated pits (cp) are not labeled by the tracer. (*B*) The probe is endocytosed in a multivesicular body (mv). The intercellular junction (j) is not permeated by the tracer. (*C*) Large vesicles (e) (most likely of the endosomal compartment) are decorated. (*D*) At 15 min, several labeled plasmalemmal vesicles are present on the abluminal front (av). (*E*) In this segment, almost every vesicle associated with the abluminal front (av) appears as discharging its contents into the subendothelial space (arrow). At j, an intercellular junction containing tracer in its luminal infundibulum (arrowhead) is observed. (*F*) In some areas, the complex (arrow) has reached the subendothelial extracellular compartment. l, Lumen; ss, subendothelial space; bl, basal lamina. (×75,000.)

association of diabetes with increased levels of fibrinogen, an important risk factor for vascular complications (30–32), the ability of infused AGE albumin to elevate mRNA levels of IL-6, a cytokine linked to fibrinogen synthesis (33–35), was studied. Infusion of AGE albumin into normal mice led to an increase in IL-6 transcripts, compared with control animals infused with native albumin (Fig. 3*A*, lanes 3 and 2, respectively). The migration of the PCR band amplified from AGE albumin-treated mice was identical to that induced by exposure of animals to bacterial lipopolysaccharide, a known inducer of IL-6 (33) (Fig. 3*A*, lane 6). Southern blotting of these PCR products with an oligonucleotide probe for murine IL-6 confirmed the identity of the above amplicons (Fig. 3*B*, lanes 3 and 6, respectively). The increase in IL-6 transcripts in response to AGE albumin was blocked by pretreatment of mice with anti-RAGE IgG, whereas nonimmune IgG was without effect (Fig. 3 *A* and *B*, lanes 4 and 5, respectively). Heat treatment of these preparations abrogated their ability to induce IL-6 (data not shown).

DISCUSSION

Our data indicate that AGE albumin present in the intravascular space interacts with endothelial RAGE, resulting in its removal from the plasma and subsequent endocytosis and transcytosis. Cell-bound AGEs can modulate cellular properties, as occurs after AGE-mediated activation of transcription factor NF-κB (36), generation of tumor necrosis factor and IL-1 (28), and induction of IL-6 mRNA (37). In addition, some of the AGE albumin is transferred across the endothelium by transcytosis, depositing ligand in the subendothelium where AGEs can potentially form crosslinks altering basement membrane structure and function (2), as well as interacting with other cells in the subendothelial space that bear RAGE. Subsequently, recruitment of intracellular second-messenger pathways and effector mechanisms in response to AGE engagement of RAGE could be initiated by signals in the cytosolic tail of the receptor or, in part, by the natur of the ligand itself, as AGEs have been shown to generate reactive oxygen intermediates (38–40).

Since AGEs, though a diverse class of structures, represent the final and irreversible consequence of glycation and oxidation of proteins and lipids (1–8), elucidation of a major cellular acceptor site, such as RAGE, could provide insights into the pathogenesis of disorders in which they accumulate. Although future studies employing reagents which prevent AGE–RAGE interaction over longer periods of time will be

FIG. 3. Infusion of AGE albumin induces IL-6 mRNA as shown by PCR analysis. Mice were infused with either saline alone (0), native albumin (250 μg per animal) or AGE albumin (250 μg/animal) alone, or AGE albumin in the presence of anti-RAGE IgG or nonimmune IgG (40 μg per animal). Other animals received lipopolysaccharide (LPS, 100 μg per animal, i.v.). *B* shows Southern hybridization of the products obtained in *A* with ^{32}P-labeled oligonucleotide probe for murine IL-6. *C* shows PCR product obtained with β-actin-specific primers. Migration of DNA size (bp) markers (New England Biolabs) is shown at left.

required to dissect the possible contribution of RAGE to vascular dysfunction, the current experiments establish that AGEs in the intravascular space recognize RAGE on the vessel wall as a major cell-associated target.

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